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Molecular Analysis of a Metalloprotease from *Proteus mirabilis*†

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Proteus mirabilis **is known for its ability to differentiate from swimmer to swarmer cells, a process crucial for the pathogenesis of these bacteria during urinary tract infections. Among the many virulence factors produced during swarmer cell differentiation is an extracellular metalloprotease. A cosmid containing a large fragment of *P. mirabilis* chromosomal DNA was obtained by measuring protease expression in recombinant *Escherichia coli*. The recombinant and native enzymes were purified to over 95% homogeneity from culture supernatants by use of phenyl-Sepharose affinity chromatography and found to be identical. The activity of the 55-kDa enzyme was stimulated by divalent cations ($Ca^{2+} > Mg^{2+}$) and inhibited by a chelator of these cations. The enzyme possesses substrate specificity for both serum and secretory forms of immunoglobulin A1 (IgA1) and IgA2 as well as IgG and, unlike classic IgA proteases, digested to completion both human and mouse IgA. Following subcloning, a 5-kb DNA fragment encoding recombinant protease activity was identified by insertional mutagenesis with Tn*5*. Four open reading frames were identified within this 5-kb region by limited nucleotide sequence analysis of DNA flanking the transposon. The nucleotide and deduced amino acid sequences of the metalloprotease structural gene (*zapA*) were obtained. Computerized homology studies revealed that the *P. mirabilis* metalloprotein is a member of the serralysin family of proteases and may be part of an operon comprising genes encoding an ATP-dependent ABC transporter in addition to the metalloprotease. The relevance of the metalloprotease to swarmer cell differentiation and pathogenicity is discussed.**

Proteus mirabilis is a bacterium that is often found in soil, water, and the intestinal tract of many mammals, including humans. This dimorphic bacterium can undergo dramatic morphological and physiological changes in response to growth on surfaces or in viscous environments. These changes are ultimately required to produce the multicellular motile behavior that is characterized by flagellum-assisted swarming motility over nutrient agar media. In the process of swarming, the bacteria differentiate from short, vegetative swimmer cells to elongated, highly flagellated forms referred to as swarmer cells. It is well established that inhibition of wild-type flagellar-filament rotation is critical in the induction of the differentiation process (3, 11, 49). Differentiation requires chemical signals as well as the physical signals derived from the inhibition of flagellar rotation. Glutamine is the major extracellular signal, which is sensed by a specific transduction mechanism that is independent of the cellular and nutritional amino acid uptake system (3).

P. mirabilis is not a common cause of urinary tract infection (UTI) in the normal host. Surveys of uncomplicated cystitis or acute pyelonephritis show that *P. mirabilis* is involved in only a few cases (49). However, *P. mirabilis* infects a much higher proportion of patients with complicated urinary tracts, that is, those with functional or anatomical abnormalities or with chronic instrumentation, such as long-term urinary catheterization. In these patients, *P. mirabilis* not only causes cystitis and acute pyelonephritis but also characteristically leads to the production of urinary stones, which complicates further the problems associated with the urinary tract (49).

P. mirabilis possesses a host of potential virulence factors that may aid its pathogenesis. The two major ones, flagella and urease, have been investigated most thoroughly (11, 14, 32, 36, 37, 50), but other proposed or established virulence factors, including hemolysin, four distinct types of fimbriae, amino acid deaminase, and a protease activity, may also be important. The expression of these virulence factors is coordinately regulated through a global swarmer cell regulon (4). Indeed, evidence of coordinately regulated virulence components implicates the differentiated swarmer cell as a prerequisite for the colonization and invasion of host tissue. Allison et al. (1, 2) have shown that differentiation is coupled to the ability of *P. mirabilis* to be internalized by human uroepithelial cells in vitro. Internalization is reduced, but not eliminated, in mutants that are defective in swarming.

Among the virulence components known to be coordinately regulated with cellular differentiation is a metalloprotease activity. Many strains of *P. mirabilis* produce an extracellular protease, referred to as an immunoglobulin A (IgA) protease, that is EDTA sensitive and different from other IgA proteases in that it cleaves the IgA heavy chain outside the hinge region (45, 46, 47, 57). The purified protease has been shown to be a composite of a single band and a double band (53 and 50 kDa, respectively) when analyzed under denaturing conditions (46). All three bands were demonstrated to be proteolytically active and had specificity for both serum and secretory forms of IgA1 and IgA2 as well as IgG. Although its role in *P. mirabilis* virulence has not been directly confirmed, immunoblotting of urine from patients who had a *P. mirabilis* UTI showed that 64% of the specimens with IgA produced IgA heavy-chain fragments identical in size to those formed when purified IgA was degraded by pure protease (57). Thus, it is likely that the protease is produced and is active upon urinary tract IgA during *Proteus* UTI.

In this paper, we report on the biochemical characterization in addition to the genetic and sequence analysis of a recombinant metalloprotease from *P. mirabilis* with properties similar to those of the previously described IgA protease.

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MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. *P. mirabilis* BB2000 (wild type, rifampin resistant [Rf^r]) (13) was used as the source of the extracellular metalloprotease. *Escherichia coli* DH5α [F$^-$ φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17* (r_K^- m_K^+) *deoR thi-1 supE44* λ$^-$ *gyrA96 relA1*) was used as a general host strain. *E. coli* CC118 [*araD139* Δ*ara leu7697* Δ*lacX74* Δ*phoA20 galE galK thi rpsE rpoB argE*(Am) *recA1*] was used as a host strain for Tn*5* mutagenesis with λ467 (*b*221 *rex*::Tn*5* *c*I857 *O*am29 *P*am80). LB medium (consisting of 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl [each per liter]), NZCM medium (prepared with 10 g of NZ amine [Scheffield Products, Memphis, Tenn.], 1 g of Casamino Acids, 5 g of NaCl, and 2 g of $MgSO_4$ [each per liter], and with the pH adjusted to 7.0 with NaOH), or terrific broth (consisting of 12 g of tryptone, 24 g of yeast extract, and 4 ml of glycerol [each per liter], sterilized and cooled, and with 100 ml of sterile 0.17 M KH_2PO_4–0.72 M K_2HPO_4 added) was used in experiments with *E. coli* and *P. mirabilis* as specified. When solidified medium was necessary, 15 g of agar (Difco) was added per liter of medium. *P. mirabilis* was maintained on LSW$^-$ agar (consisting of 10 g of tryptone, 5 g of yeast extract, 0.4 g of NaCl, 5 ml of glycerol, 20 g of Bacto Agar, and 1,000 ml of distilled H_2O), which phenotypically inhibited swarming motility (13). Protease activity was detected by using skim milk agar (consisting of 15 g of peptone and 15 g of agar per liter, sterilized and cooled, to which 10 g of sterilized dry milk powder [Carnation] in 200 ml of distilled H_2O was added). Antibiotics (Sigma) were added to media at the following concentrations (in micrograms milliliter^{-1}): ampicillin, 100; chloramphenicol, 40; kanamycin sulfate, 80; rifampin, 100. The chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Gold Biochemical) was used at a concentration of 40 μg ml^{-1}.

Recombinant DNA methods. Recombinant DNA techniques, including restriction endonuclease digestion, ligation, and transformation, were carried out by standard methods (6, 55, 62). The construction, analysis, and use of the *P. mirabilis* chromosomal DNA cosmid library have been reported previously (12, 14). Cosmid pCOS17-4-25, acquired from this bank, is pCAMR3 (RK2, Mob$^+$, chloramphenicol resistant [Cm^r] [14]) carrying a 27-kb insert of *P. mirabilis* chromosomal DNA. Plasmid pCW101 is an *Eco*RI subclone of pCOS17-4-25 on pBluescript SK+ (Stratagene) and is ampicillin resistant (Ap^r).

Skim milk agar screening of protease activity. Bacteria to be tested for protease activity were either streaked or spot inoculated on the surface of skim milk agar and incubated at 37°C for 24 to 72 h. Protease activity was identified as a distinct clearing of the milk around the colony.

Purification of protease by phenyl-Sepharose affinity chromatography. The protease was purified by affinity chromatography by a modification of the method described by Loomes et al. (47). Bacterial cultures were incubated overnight at 37°C in 1-liter cultures of LB medium or LB medium amended with the appropriate antibiotic. Cells and debris were removed by centrifugation at 10,000 × *g* for 30 min at 4°C. The protease-containing supernatants were filtered through 0.45-μm-pore-size filters (Micron Separations Inc.), and the filtrates were loaded at a rate of 1 ml/min at 4°C onto columns (2.5 by 50 cm; total bed volume, 240 ml) of phenyl-Sepharose (Pharmacia) equilibrated in 50 mM Tris-HCl (pH 8.0). The columns were then washed with 10 column volumes of 50 mM Tris-HCl buffer (pH 8.0) or until unbound material was removed. Bound protease was then eluted with 50 mM Tris buffer (pH 11). The pH of the fractions (3.5 ml) of eluted protease was adjusted to 8.0 with HCl after collection and pooled. Purified protease from *P. mirabilis*, but not from recombinant *E. coli*, contained a nonprotein yellow impurity. This contaminant was removed by anion-exchange chromatography with DEAE 52 cellulose (Whatman) contained within a column (1 by 4 cm; Isolabs) to which a step gradient of 0 to 0.5 M NaCl in 50 mM Tris (pH 8.0) was applied. The pure protease eluted from this column was devoid of the yellow impurity, which remained bound to the column, was dialyzed against 50 mM Tris (pH 8.0), and was stored at 4°C until further use.

Protein analyses. Protein concentration was determined by use of the microbicinchoninic acid protein assay reagent as recommended by the manufacturer (Pierce Chemical Co.). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (39) with modifications reported previously (11, 12) by use of slab gels consisting of a 10 to 20% acrylamide gradient resolving gel (Ready Gels; Bio-Rad). Reduced samples were boiled with an equal volume of 0.1 M Tris–2% SDS–80 mM β-mercaptoethanol–0.025% (wt/vol) bromophenol blue (stop buffer) for 5 min and then loaded on the gel. After electrophoresis at 100 V until the dye front reached the bottom of the gel, the gels were stained with Coomassie brilliant blue and destained by the method of Fairbanks et al. (28). The molecular weights of proteins were estimated from their mobilities relative to a set of commercially available proteins (Diversified Biotech, Newton Centre, Mass.). These standard proteins included phosphorylase *b* (95.5 kDa), glutamate dehydrogenase (55.0 kDa), ovalbumin (43.0 kDa), lactate dehydrogenase (36.0 kDa), carbonic anhydrase (29.0 kDa), lactoglobulin (18.4 kDa), and cytochrome *c* (12.4 kDa).

Azocaseinase assay. Enzymatic release of azo dye from azocasein was used to measure protease activity by the method of Loomes et al. (46). Briefly, a 0.5% (wt/vol) azocasein solution was prepared by dissolving azocasein in 2 mM $CaCl_2$–50 mM Tris (pH 8.0). Insoluble material was removed by centrifugation, and the azocasein solution was stored at 4°C for use within 72 h. To analyze relative protease activity, protease was diluted as required to a final volume of 100 μl to which was added 900 μl of the 0.5% azocasein solution. Standard assay conditions were incubation at 37°C for 30 min. After incubation, the reaction was terminated by the addition of 250 μl of 15% (wt/vol) trichloroacetic acid. After the sample was left standing for 5 min, the unhydrolyzed azocasein precipitate was removed by centrifugation at 14,000 × *g* for 5 min. The clear supernatant was removed, and the A_{440} relative to that of a buffer control was determined. One unit of protease was defined as the activity which produced a change of 0.1 absorbance unit and was calculated from the following equation: units of protease per microgram of protein = (A_{440} × dilution factor)/(protein concentration × 0.1 absorbance unit × volume of enzyme) (46).

Measurement of enzyme activity as affected by divalent cations was done with the same reaction conditions except that either H_2O or 2 mM $MgCl_2$ was used in place of $CaCl_2$. Divalent cations were removed from the medium by the addition of 10 mM EDTA that was added as a stock of 0.5 M EDTA (pH 8.0).

Substrate specificity analysis. The substrate specificity was assessed by incubating 0.1 to 15 U of protease with 5 to 10 μg of protein substrate in 50 mM Tris-HCl (pH 8.0) buffer containing 2 mM $CaCl_2$ in a final volume of 20 μl. After incubation at 37°C for the appropriate time, the reaction was stopped by adding 2 volumes of 2× Laemmli stop buffer and boiling for 5 min. The samples were then analyzed by SDS-PAGE. The protein substrates used were bovine serum albumin fraction V (BSA), casein, cytochrome *c*, phosphorylase *b*, and ovalbumin (albumin from chicken egg), all of which were obtained from Sigma Chemical Co. Human serum (plasma) IgA1, IgA2, IgG, and myeloma (secretory) IgA were obtained from Calbiochem. Mouse IgA was a gift from Harry Mobley (Division of Infectious Diseases, University of Maryland Medical School, Baltimore). *P. mirabilis* flagellin was prepared as described previously (12, 14).

Tn*5* mutagenesis. Mutagenesis of pCW101 by λ467::Tn*5* was performed as described previously (7, 8, 61) with minor modifications. The recipient *E. coli* CC118 harboring pCW101 was grown overnight at 37°C in NZCM medium containing ampicillin. The culture was diluted 1:20 into 50 ml of fresh NZCM medium and grown at 37°C to an optical density at 600 nm of 0.8 to 1.0. In each mutagenesis, from 1 to 100 μl of bacteriophage stocks (prepared by the method of Sambrook et al. [55]) was added to 1 ml of cells to give a multiplicity of infection of ca. 1. Phage adsorption was for 40 min at room temperature; the addition of 2 ml of LB medium containing ampicillin followed. The kanamycin expression period was for 2 h at 30°C, after which 2 ml of each 3-ml culture was transferred to a tube already containing 3 ml of Terrific Broth with ampicillin and kanamycin. Growth was continued overnight at 37°C, and 200 μl from each culture was inoculated into 5 ml of fresh terrific broth containing ampicillin and kanamycin the following day. These cultures were grown at 37°C for 24 h. A 2-ml aliquot was used for a miniplasmid preparation by an alkaline lysis procedure. The DNA obtained was used to transform *E. coli* DH5α, and selection was on LB medium plates with ampicillin (for maintenance of pCW101) and kanamycin (to ensure the presence of the Tn*5* on the plasmid). Mutations affecting protease production in *E. coli* were screened on skim milk agar medium as described above. Two hundred *E. coli* transformants were isolated from five independent mutageneses, plasmid preparations were made from the cultures, and the site of each Tn*5* insertion in pCW101 was identified by restriction fragment analysis.

Nucleotide and deduced amino acid sequence analysis. Since the insertion site of each Tn*5* had been mapped by restriction analysis, digestion with *Sal*I and *Eco*RI or *Sal*I alone allowed identification of clones containing the right- and left-hand fragment for each Tn*5* insertion. An oligonucleotide homologous to the end of the Tn*5* arm, i.e., 3′-GTTCATCGCAGGACTTG-5′ (61), was used as the primer for double-stranded plasmid DNA sequencing by the dideoxy chain termination method (56). Reactions were run with reagents from the Prism Ready Reaction Dye Deoxy Termination Kit (Applied Biosystems) in conjunction with *Taq* polymerase. Reactions were run on a model 373A DNA sequencer (Applied Biosystems). The sequence of the *zapA* gene was completed with oligonucleotide primers complementary to the sequence obtained as described above to extend sequences from the Tn*5* end. The complete nucleotide sequence was determined in both directions and has been stored in the GenBank repository of nucleotide sequences under accession number U25950.

Nucleotide and deduced amino acid sequences were analyzed and compared with other known sequences in the GenBank library of DNA sequences by use of Genetics Computer Group (Madison, Wis.) computer programs and the BLASTN and BLASTX programs developed by the National Center for Biotechnology Information (5, 24, 31).

Materials and reagents. All reagents were of the highest purity available. Components of bacteriological media were purchased from Difco. Restriction endonucleases and DNA-modifying enzymes were obtained from either New England Biolabs, Boehringer Mannheim Biochemicals, or Promega and used as described in the supplier's recommendations.

RESULTS

Cloning of an extracellular protease activity from *P. mirabilis*. As has been demonstrated for most strains of *P. mirabilis* (47), strain BB2000 produces an extracellular protease that can be detected when these bacteria are placed on media containing a suitable substrate such as skim milk agar (data not

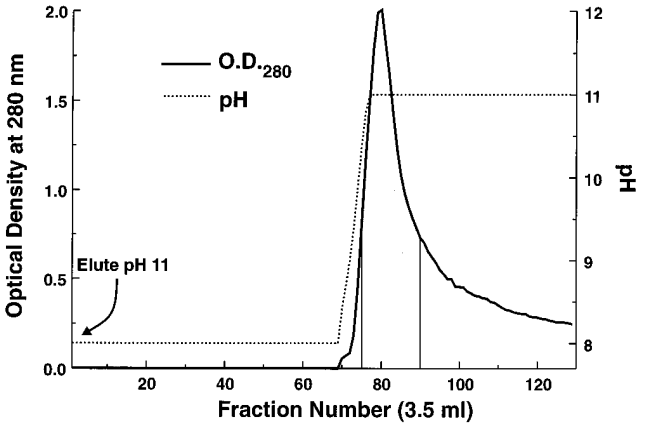

FIG. 1. Purification of recombinant protease from culture supernatant by phenyl-Sepharose column chromatography. The elution profile of fractions collected after the column was washed with 10 column volumes (2,500 ml) of 50 mM Tris (pH 8.0) to remove any unbound material is shown. Fraction 1 represents the first fraction (3.5-ml volume) collected after the buffer was changed to 50 mM Tris (pH 11.0) as required to desorb the protease. The shaded area under the peak represents the pooled fractions containing protease activity as measured by the azocasein assay. The protein absorbance profile at an optical density at 280 nm (OD_{280}) is indicated as a solid line, while the pH of the fractions is represented by a dotted line.

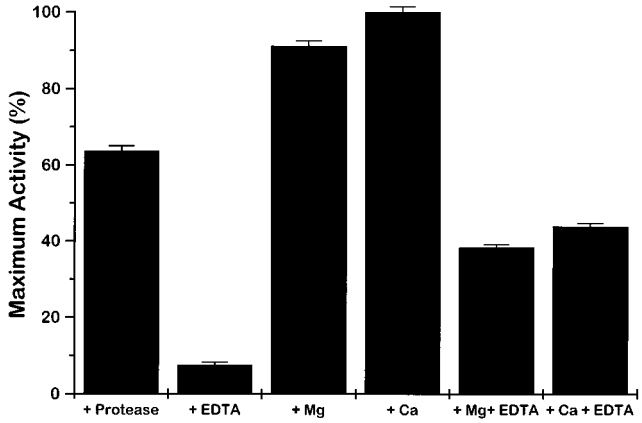

FIG. 2. Effect of divalent cations on protease activity. Recombinant protease (50 U; 1 μg) was added to azocasein in 50 mM Tris (pH 8.0) to which was added either 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM EDTA, or H_2O. After 30 min of incubation at 37°C, the samples were processed as indicated in Materials and Methods and the units of protease were determined. Triplet measurements were averaged, and the standard error of the mean was calculated. The percentage of activity compared with that of the sample giving the greatest activity (protease plus 2 mM $CaCl_2$) was determined and plotted. The columns represent, from left to right, protease alone, protease plus EDTA, protease plus $MgCl_2$, protease plus $CaCl_2$, protease plus $MgCl_2$ and EDTA, and protease plus $CaCl_2$ and EDTA.

shown). We took advantage of this to screen a cosmid library of *P. mirabilis* genomic sequences for recombinant *E. coli* that was capable of producing zones of clearing on skim milk agar. Of approximately 4,000 cosmid clones analyzed, 24 recombinant colonies degraded the skim milk to various degrees. Restriction endonuclease cleavage and DNA fragment analyses were done on isolated cosmid DNA from these protease-positive (Prt^+) clones. This analysis demonstrated that the cosmids overlapped each other, and as a result, one cosmid, pCOS17-4-25, bearing a ca. 27-kb insert, was chosen for further analysis.

The region of genomic DNA on pCOS17-4-25 was reduced in size by digestion with *Eco*RI and ligation to pBSSK+ (Stratagene). Several Prt^+ clones were picked after transformation of *E. coli* DH5α, and their plasmid DNA was compared by restriction enzyme cleavage and agarose gel DNA fragment analysis. Since all of the subclones contained an identical insert, one representative plasmid, pCW101, bearing a 9-kb insert, was chosen for further study.

Biochemical characterization of the recombinant protease enzyme. The recombinant extracellular protease was purified by affinity chromatography to phenyl-Sepharose as described by Loomes et al. (47). Overnight liquid cultures of DH5α harboring pCW101 were incubated at 37°C in LB medium containing ampicillin. When protease preparations from overnight cultures were applied to columns of phenyl-Sepharose equilibrated in 50 mM Tris-HCl (pH 8.0), protease activity bound to the column. After thorough washing of the column to remove other proteins and contaminants, the bound protease was eluted with 50 mM Tris-HCl (pH 11.0). Short-term exposure to pH 11.0 did not affect protease activity; however, to maintain maximal activity, the pH of the eluted fractions was adjusted to pH 8.0 after collection.

A typical result is presented in Fig. 1, which shows the phenyl-Sepharose affinity chromatography of a culture supernatant of DH5α harboring pCW101. Identical results were obtained when either DH5α carrying pCOS17-4-25 or wild-type *P. mirabilis* supernatants were used (data not shown). Most of the nonprotease proteins were removed from the column by washing at pH 8. Subsequent application of buffer at pH 11 eluted a single peak of UV-absorbing material (Fig. 1). When *P. mirabilis* culture supernatants were used, this peak also contained a yellow pigment that was removed by anion-exchange chromatography on a DEAE 52 cellulose column by a single-step application of 0.5 M NaCl incorporated in the 50 mM Tris-HCl buffer (pH 8.0). This purification scheme typically provided between 200 and 500 μg of protein per liter of culture supernatant. The relative protease activity of the recombinant enzyme was ca. 15 to 17 U/μg of protein as measured by azocasein digestion (see Materials and Methods).

The effects of the divalent cations Mg^{2+} and Ca^{2+} and the chelating agent EDTA on the activity of the recombinant protease are presented in Fig. 2. The addition of either Mg^{2+} or Ca^{2+} increased protease activity, while removal of divalent cations by chelation with EDTA was inhibitory. The activity of the enzyme in the absence of added divalent cation (ca. 65% of maximal activity) suggests that, as purified, the protein has divalent metal ions already bound to it. These results indicate that the recombinant protease from *P. mirabilis* is a metalloprotease. Identical results were obtained when the native *P. mirabilis* enzyme was used. These results agree with the results obtained by Loomes et al. (47), who have reported that numerous *Proteus* strains secrete an EDTA-sensitive metalloprotease (58, 59).

The only extracellular protease from *P. mirabilis* that has been biochemically purified and characterized is a composite protein of a single band and a double band (53 and 50 kDa under denaturing conditions) that digests the heavy chain of human IgA to produce a major fragment of 47 kDa and a minor fragment of 37 kDa (46, 47, 57). The recombinant metalloprotease was therefore analyzed for molecular weight, reaction kinetics, and substrate specificity to characterize the cloned enzyme. Figure 3 shows the results of a time course of the recombinant metalloprotease digestion of human serum IgA1. As can be observed in Fig. 3, lane 2, the recombinant metalloprotease preparation is highly purified, being composed of a single protein with a molecular mass of 55 kDa.

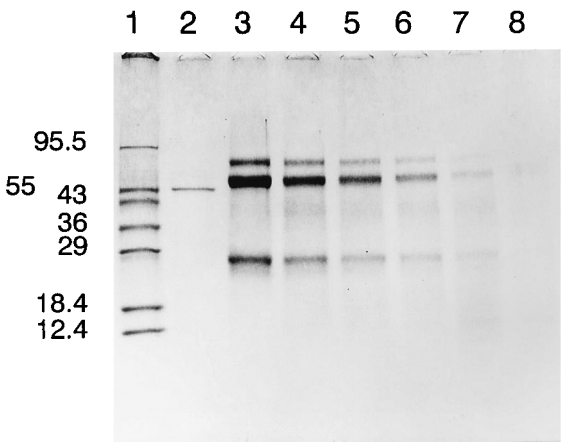

FIG. 3. Time course of metalloprotease digestion of human serum IgA1. Recombinant metalloprotease (5 U; 0.1 μg) was reacted with 5 μg of human serum IgA1 in 2 mM $CaCl_2$ buffered with 50 mM Tris (pH 8.0). The reaction was performed at 37°C for either 0 h (lane 3), 1 h (lane 4), 2 h (lane 5), 4 h (lane 6), 8 h (lane 7), or 16 h (lane 8). Protein molecular weight standards are shown in lane 1 (in thousands), and 1 μg of purified recombinant protein is shown in lane 2.

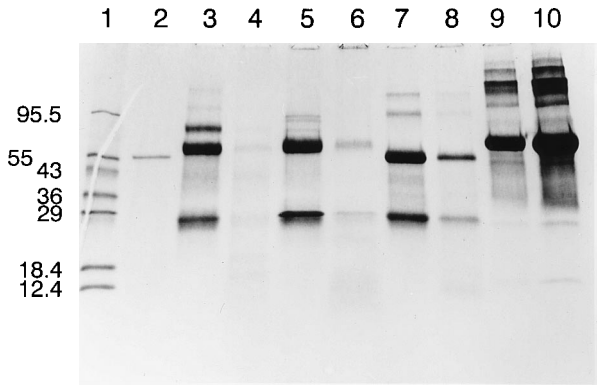

FIG. 4. Substrate specificity of the metalloprotease. Recombinant metalloprotease (5 U; 0.1 μg) was incubated with 5 μg of either human serum IgA1, mouse IgA, human IgG, or BSA for 8 h at 37°C. Protein molecular weight standards (in thousands) are shown in lane 1, and 1 μg of purified recombinant protease is shown in lane 2. The samples are human IgA1 (lane 3), human IgA1 plus enzyme (lane 4), mouse IgA (lane 5), mouse IgA plus enzyme (lane 6), human IgG (lane 7), human IgG plus enzyme (lane 8), BSA (lane 9), and BSA plus enzyme (lane 10).

Such analyses suggest that these preparations contained at least 95% pure metalloprotease. The presence of additional bands as demonstrated by others (47) was not detected even when more sensitive silver staining methods and linear SDS-PAGE were used (data not shown). This recombinant enzyme degrades human IgA1 in a time-dependent manner (Fig. 3, lanes 3 to 8), resulting in complete digestion of the IgA1 substrate into numerous smaller fragments. Discrete product bands at 47 and 37 kDa were not observed, although ill-defined bands of that general size were observed after 1 h of digestion (Fig. 3, lane 4). Additionally, at 8 h (Fig. 3, lane 7), two faint bands at 10 and 15 kDa were seen; however, these bands were absent after 16 h of incubation (Fig. 3, lane 8). The identical pattern of digestion was seen for both the recombinant enzyme as well as that isolated from *P. mirabilis*. These results suggest that the cloned enzyme from *P. mirabilis* BB2000 is biochemically similar, but not identical, to the protease characterized by Loomes et al. (46, 47, 57). The disagreement in relative molecular weight between this protease and the protease characterized by Loomes et al. (47) may be due to differences in experimental technique, while the lack of specific site cleavage of IgA could be due to differences between bacterial strains or the substrate and reaction conditions used by the two groups. These differences are discussed in more depth in Discussion. From these experiments, 8 h was chosen as a fixed time for incubation and analysis of the metalloprotease substrate activity.

SDS-PAGE analysis was used to examine the substrate specificity of the recombinant metalloprotease (Fig. 4). Both human serum IgA1 (Fig. 4, lanes 3 and 4) and human IgG (Fig. 4, lanes 7 and 8) were digested by the recombinant metalloprotease. Additionally, mouse IgA (Fig. 4, lanes 5 and 6) is also a substrate for the recombinant metalloprotease, an observation that is uncommon among IgA proteases that classically are limited to IgA from humans and related primates (38). The recombinant protease also digested human serum IgA2 and secretory IgA as well as casein (data not shown), in agreement with the observations of Loomes et al. (45) and Senior et al. (57). The enzyme did not have activity against BSA (Fig. 4, lanes 9 and 10), cytochrome *c*, *P. mirabilis* flagellin, ovalbumin, or phosphorylase *b*, demonstrating that the specificity of the recombinant metalloprotease is limited to immunoglobulins, an observation supported by the work of Loomes et al. (45) and Senior et al. (57). Identical results were obtained when the native *P. mirabilis* enzyme was used in these experiments, indicating that the cloning process did not result in a loss of enzymatic specificity or activity. Identical results were also obtained with both phenyl-Sepharose-purified enzyme and $(NH_4)_2SO_4$-concentrated crude culture supernatants, indicating that the method of purification did not change the specificity of the enzyme (data not shown).

Tn*5* mutagenesis and analysis of flanking DNA. The recombinant plasmid pCW101, used throughout the previous biochemical characterization, possesses a 9-kb insertion of *P. mirabilis* genomic DNA. This region is sufficiently large to encode several genes. We wanted to localize the region of this DNA that was essential for the recombinant metalloprotease activity (Prt^+ phenotype). Attempts to reduce the 9-kb region through further subcloning were unsuccessful as a result of a paucity of convenient restriction endonuclease sites. As an alternative, we chose to use transposon Tn*5* mutagenesis (7, 8) to identify the region responsible for producing the metalloprotease activity. An *E. coli* recombinant with pCW101 (Prt^+) was infected with λ::Tn*5*, and infected cultures were plated on a medium containing kanamycin to select for strains with transposon insertions (see Materials and Methods). The resulting kanamycin-resistant (Km^r) colonies contained insertions in either the recombinant plasmid or the *E. coli* genome. Plasmid DNA was purified from pooled cultures of the Km^r colonies and reintroduced by transformation into an *E. coli* host that was then subjected to selection for ampicillin and kanamycin resistance to obtain recombinant plasmids containing transposon insertions. To identify the location of the region of cloned DNA encoding production of the metalloprotease, *E. coli* recombinants with mutated plasmids were screened for those which were defective in producing zones of clearing on skim milk agar medium (Prt^-). In total, 35 Prt^- mutants were obtained from a pool of 200 Ap^r and Km^r mutants.

The location of each of the Tn*5* insertions in pCW101 yielding a Prt^- phenotype was determined by restriction endonuclease cleavage of purified plasmid DNA and subsequent DNA fragment analysis and mapping. A schematic map showing the insertion points of the transposon insertions is presented in

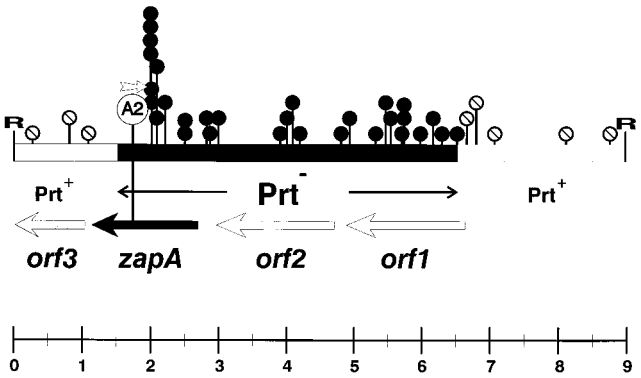

FIG. 5. Transposon mutagenesis of the metalloprotease genetic locus. The DNA required for metalloprotease activity harbored on pCW101 was defined by Tn*5* mutagenesis. Transposon insertions that resulted in a Prt$^-$ phenotype (closed circles) are located in one 5-kb region of the *P. mirabilis* DNA as indicated by the shaded area on the 9-kb clone. Cross-hatched circles depict a Tn*5* insertion resulting in a Prt$^+$ phenotype. Limited nucleotide sequence analysis indicates that a minimum of four ORFs are contained within this region (see text for details). The direction of transcription of each ORF is from right to left as indicated by the arrows. Transposon A2 that is inserted within the metalloprotease structural gene is shown. The arrow above A2 indicates the direction of the IS*50L* end of the Tn*5* used for initial nucleotide sequence analysis. R, *Eco*RI.

Fig. 5. Transposon insertions within a 5-kb region of *P. mirabilis* DNA resulted in a Prt$^-$ phenotype, while Tn*5* insertions outside of this 5-kb region were Prt$^+$. The length of DNA affected suggests that more than one gene is necessary for protease activity in recombinant *E. coli.*

The strategy used to sequence the DNA region encoding the metalloprotease activity employed Tn*5* insertions that provided well-spaced, defined restriction fragments terminated with a unique sequence (part of the Tn*5* IS*50L* arm) to which one primer oligonucleotide could be hybridized for nucleotide sequencing (61). Additionally, prior results from limited nucleotide sequencing of flanking *P. mirabilis* DNA from such Tn*5* insertions have shown that as little as 100 to 200 bases of sequence can reliably be used in computerized homology searches of GenBank and related databases to determine the *P. mirabilis* gene mutated by the transposon (15). Such analysis of the 5-kb Prt$^-$ region revealed the presence of at least four open reading frames (ORFs) as indicated in Fig. 5. These ORFs are all oriented in the same direction and may be grouped in one operon.

Limited nucleotide sequence was obtained from transposon insertions in ORF1 and ORF2 and revealed that neither of these genes has homology to known proteases (data not shown). These ORFs may be important for protease activity because Tn*5* insertions in either result in a Prt$^-$ phenotype. Conversely, polar effects due to Tn*5* insertions in a gene upstream of the protease gene in an operon could also produce the observed phenotype. Homology searches of the GenBank database, however, have indicated that this region bears sequences with strong similarity to the ATP-binding-cassette (ABC) transporter family of proteins (34). For example, the partial deduced amino acid sequence of ORF1 is homologous to *Serratia marcescens* PrtD, while a portion of the deduced amino acid sequence of ORF2 is homologous to PrtE from the same bacterium (44). This ORF possesses the classic ABC transporter signature, AHGEGLSGGQKQRIALARALYGD PT. Highly homologous signature sequences have been found in several proteins known to bind ATP and which are involved in various transport processes in prokaryotes and eukaryotes, including the secretion of metalloproteases from *S. marcescens* (43, 44), *Erwinia chrysanthemi* (21, 22), and *Pseudomonas aeruginosa* (26). The complete analysis of the nucleotide and deduced amino acid sequences for this region is the subject of a future report (unpublished data).

Sequence analysis of the protease structural gene (*zapA*). The flanking DNA from one of the transposon insertions (identified as A2 in Fig. 5) has strong homology to a very large group of closely related metalloproteases from both prokaryotes and eukaryotes. This ORF, designated *zapA*, was thus likely to encode the structural gene for the *P. mirabilis* metalloprotease, and a complete nucleotide and deduced amino acid sequence analysis was therefore performed. These data are presented in Fig. 6. The nucleotide sequence has been stored in the GenBank repository of nucleotide sequences and given the accession number U25950.

In total, 1,940 bp of nucleotide sequence were analyzed with the A2 Tn*5* insertion (Fig. 5) flanking DNA (located at nucleotide 900 in Fig. 6) as a starting point and then by filling in the remaining sequence with pCW101 as template DNA and oligonucleotide primers to extend the sequence. The G+C content (34.8%) and the codon usage of each ORF in this region are typical for *P. mirabilis* (14, 19). Analysis of this DNA reveals that it contains one complete ORF (*zapA*) of 1,473 bp whose coding region extends from nucleotide 223 to 1,695. A near-consensus ribosome binding site (60) is located 5′ of the ATG start codon for this gene. The stop codon of this ORF is followed by an area of strong stem-loop formation, with the resulting structure estimated to have a free energy of −13.6 kcal (ca. −57 kJ) (67). A second ribosome binding site is found 5′ of a second ORF that is located in the same reading frame downstream from *zapA*. Neither of these genes appears to have distinct consensus promoter regions, perhaps suggesting that they are members of a larger operon.

As indicated earlier, the analysis of the deduced amino acid sequence of the 1,473-bp ORF provides convincing evidence that this is the structural gene of the secreted metalloprotease. The deduced amino acid sequence of ZapA predicts an acidic protein (pI = 4.30) composed of 491 residues with a total molecular mass of 53,999.69 Da. Interestingly, ZapA is glycine rich (11.2 mol%). Since ZapA is exported to the exterior of the cell, we examined the N-terminal domain of the deduced amino acid sequence for homology to signal sequences of other well-characterized secreted proteins (66). Such an N-terminal signal sequence was not evident on ZapA. Instead, computerized protein homology searches comparing the sequence of ZapA with other protein sequences from both prokaryotes and eukaryotes revealed that ZapA is highly homologous to the zinc metalloprotease famdily of proteins (Fig. 7). Specifically, ZapA is a member of the serralysin (EC 3.4.24.40) family of metalloproteases that includes the proteases of *S. marcescens* (PrtSM), *E. chrysanthemi* (PrtABCG), and *P. aeruginosa* AprA. One hallmark characteristic of this group of metalloproteases is the use of a C-terminal secretion signal for export to the exterior of the cell. Four distinctive protein signature domains that support this idea were found. The first signature domain, encompassing N-178 to G-203, is homologous to the Zn^{2+} metalloprotease zinc binding region of this protein family. The location of E-187 is appropriate for this residue to act as the catalytic base (9). Additionally, the three histidines in this region, H-186, H-190, and H-196, are correctly located to function as a putative Zn^{2+} binding site. The second motif found in the serralysin family of proteases is the Met turn of metzincins (9), which in ZapA is located at T-222 to Y-226, with M-224 being the conserved residue in this motif. The last two signatures are perhaps the most relevant to the putative function of ZapA. Each of the metalloproteases in Fig. 7 is also charac-

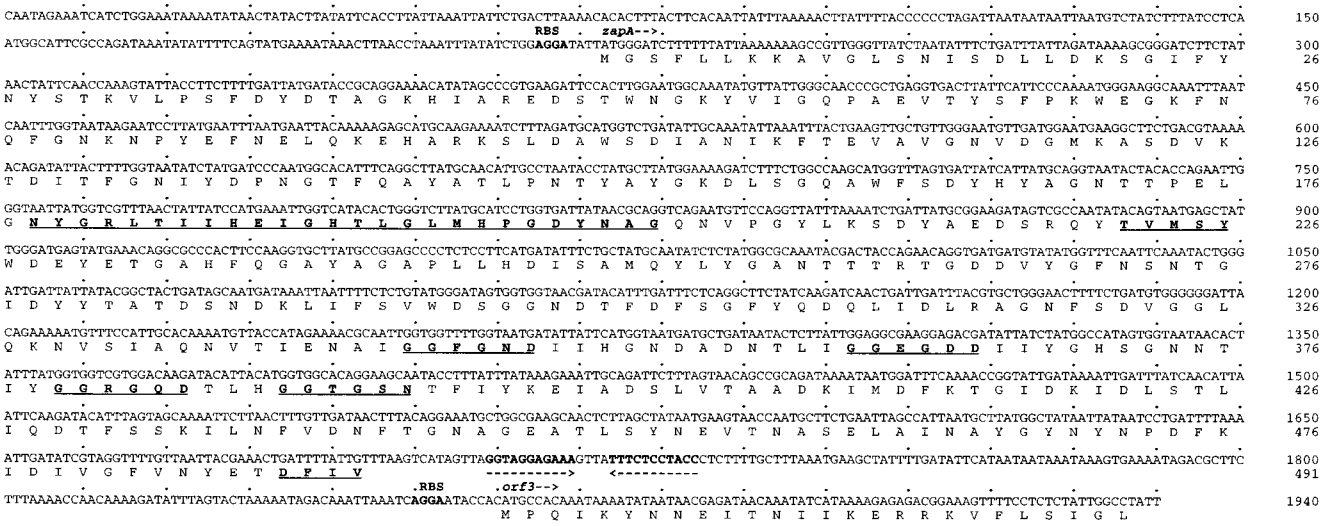

FIG. 6. Nucleotide and deduced amino acid sequences of *zapA*. The starts of the *zapA* and *orf3* coding regions are indicated above the nucleotide sequence. The directions of transcription (arrows) are from left to right, top to bottom. Potential candidates for ribosome binding sites (RBS) are indicated, as is a putative terminator region 3′ of the *zapA* stop codon. The region of dyad symmetry in the terminator is indicated by arrows beneath the nucleotide sequence. Relevant areas of the deduced amino acid sequence with peptide signature motifs are indicated by underlining the amino acid residues that are part of the motif. The zinc metalloprotease signature is located at N-178 to G-203; the Met turn of metzincins is found at residues T-222 to Y-226; four putative Ca^{2+} binding regions associated with the β roll domain of ABC transporter-dependent proteins can be found at G-343 to D-348, G-361 to D-366, G-379 to D-384, and G-388 to N-393; and last, a DXXX COOH terminal secretory signal is located at D-488 to V-491 (DFIV). These sequence data appear in the GenBank nucleotide sequence data library under the accession number U25950.

terized by having a glycine-rich COOH-terminal domain with signature motifs of a Ca^{2+} binding region of ABC transporter-dependent exported protein repeats of the motif GGXGXD, producing a β roll conformation that serves as Ca^{2+} binding sites (9). Characteristically, ZapA has three of these sites located at G-343 to D-348, G-361 to D-366, and G-379 to D-384. A fourth Ca^{2+} binding motif may also exist (G-388 to N-393), although it lacks consensus at the last residue, with asparagine substituted for aspartate. Finally, the last four residues of ZapA, DFIV, are the hallmark signature for COOH terminal secretory signals (30).

Our analysis of the *zapA* coding region and limited sequence analysis of the nucleotide sequence flanking Tn*5* insertions producing defective protease activity suggest that ZapA may be part of an operon encoding an ATP-dependent ABC transporter protease and secretory system similar to the protease operons of *S. marcescens*, *E. chrysanthemi*, and *P. aeruginosa* (34, 54, 66). On the basis of these three examples, we believe that the *zapA* gene is part of an operon of at least three other genes (Fig. 5), two of which (*orf1* and *orf2*) may encode proteins required for the specific transport of ZapA out of the cell. The function of *orf3* is not known at this time, but the deduced amino acid sequence has limited homology to that of several eukaryotic protein tyrosine kinases (data not shown).

DISCUSSION

We are interested in understanding the regulation of swarmer cell differentiation and behavior and how that process aids in the pathogenicity of *P. mirabilis*. The metalloprotease offers an interesting target for such investigations for at least two reasons. First, there is some evidence that the protease is coordinately regulated during swarming, and protease mutants (Prt^-) are defective in wild-type swarming behavior (4, 10). We hypothesize that the metalloprotease may play a role in intercellular signalling events controlling differentiation and motility, perhaps by influencing levels of glutamine or some other unidentified sensory molecule. Second, as described above, there is some suggestion that the metalloprotease is involved in pathogenicity, but its contribution to colonization and invasion of host tissue is unclear. The construction of *P. mirabilis* mutants lacking this protease would thus be one way of assessing the role of this virulence factor.

Pathogenic bacteria have developed numerous means to adapt to their host environment, often through the expression of bacterial virulence factors. In some cases, these virulence factors have the specific role to impair the host immune response. One of the virulence molecules *P. mirabilis* appears to use in such a role is a metalloprotease that has specificity for IgA and IgG. Such a protease activity has been observed in many isolates of *P. mirabilis* and *Proteus vulgaris* (47).

Bacteria producing proteolytic enzymes capable of degrading IgA are not common. Of the strains identified with IgA protease activity, the pathogens of mucosal surfaces such as *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* have been best studied (53). It is believed that IgA protease activity is a virulence factor since nonpathogenic strains of the same organisms do not produce this protease (46). The IgA proteases of these bacteria are distinguished from other proteases by their extremely narrow substrate specificity, which is restricted to the IgA1 subclass of immunoglobulins from humans, chimpanzees, and gorillas (38). The enzymes cleave the heavy chain of IgA1 at Pro-Ser or Pro-Thr bonds within a 13-amino-acid, proline-rich segment in the hinge region. This segment is not present in IgA2, which is thereby resistant.

Loomes et al. (46) have shown that the IgA protease from *P. mirabilis* is different. Numerous *Proteus* strains secrete an EDTA-sensitive protease that cleaves IgA (58, 59). The ca. 50-kDa protease purified and characterized by these workers is different from the other IgA proteases in that it appears to cleave the IgA heavy chain outside the hinge region (58) to produce a major fragment of 47 kDa and a minor fragment of 37 kDa. (A third 60-kDa product was also seen by this group

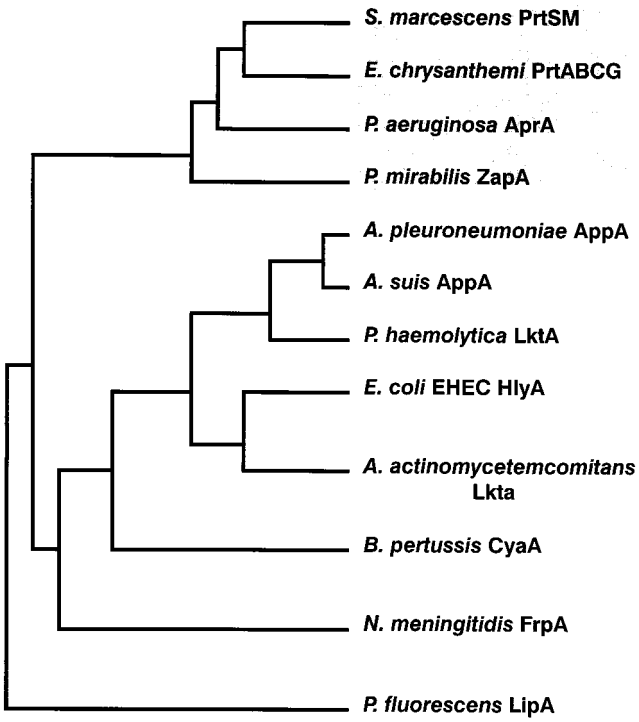

FIG. 7. Phylogenetic linkage of ZapA to the serralysin family of zinc metalloproteases. Analysis of the deduced amino acid sequence of ZapA indicates the presence of several signature peptide motifs that are held in common with many other homologous metalloproteases belonging to the serralysin family of metalloproteases, including the proteases of *S. marcescens*, *E. chrysanthemi*, *P. aeruginosa*, and (from this research) *P. mirabilis*. The serralysin family has the zinc metalloprotease domain, the Met turn of metzincins, and three or more Ca^{2+} binding motifs, and all are exported by a COOH terminal secretory signal. The serralysin family of zinc metalloproteases is in turn part of a larger set of proteins thought to function as virulence factors in several genera of bacteria. This set of virulence proteins includes *Actinobacillus* (*Haemophilus*) *pleuropneumoniae* hemolysin protein, AppA (18), *Actinobacillus suis* AppA (17), *Pasteurella haemolytica* LktA leukotoxin (20), enterohemorrhagic *E. coli* HlyA hemolysin (33), *Actinobacillus actinomycetemcomitans* LktA leukotoxin (40, 41), *Bordetella pertussis* adenylate cyclase CyaA (16), *N. meningitidis* Fe-regulated RTX cytotoxin homolog (FrpA) (64), and *Pseudomonas fluorescens* lipase LipA (27, 63). All of these proteins have the common feature of the Ca^{2+} binding GGXGXD repeat motif among other characteristics. The phylogenetic linkage map was established from nucleotide and amino acid sequences stored in the GenBank, EMBL, and Pir libraries and was analyzed with the Genetics Computer Group program PILEUP.

but was attributed to a feature of low protease activity or early degradation of the substrate [57]). This enzyme cleaved the heavy chains of serum and secretory IgA1, IgA2, and IgG but not their light chains. The enzyme also cleaves secretory component, casein, gelatin, and BSA (47).

Many of the data obtained in this study from both the native *P. mirabilis* metalloprotease and the *E. coli* recombinant enzyme agree with the findings of Loomes et al. (45–47, 57–59). The relative molecular weight, divalent cation requirement, and substrate specificity are similar to those of previous reports, suggesting that the enzyme we have analyzed and whose gene we have cloned shares features with the enzyme studied by Loomes et al. (47). On the other hand, we could not duplicate with either the native or recombinant enzyme the composite nature of the protease described by Loomes et al. (47) nor the site-specific proteolysis of IgA1, IgA2, or IgG.

The predicted molecular mass of 53,999 Da obtained from the deduced amino acid sequence of the *zapA* gene is a close match to the molecular mass of ZapA (55,000 Da) observed by SDS-PAGE, which typically has a margin of error of ca. 1,000 Da in this region of the gel. These data argue in favor of a single primary protein product of the *zapA* gene in the range of 54 to 55 kDa; however, this is not consistent with the composite structure and sizes reported by Loomes et al. (47). Several possibilities exist to explain this discrepancy. For example, our strain may be either defective in the production of one or more additional proteases or unable to correctly process the protease. This processing may be either an export function or proteolysis of preprotease molecules, as has been observed among other members of the serralysin family of proteases (21, 22, 26, 43, 44), thus lending credence to the second possibility. We are exploring both of these possibilities by constructing a $ZapA^-$ null mutant in *P. mirabilis* (unpublished data).

We have also attempted to duplicate the results of Loomes et al. (47) by altering both the specific activity of the enzyme-to-substrate ratio and the time of reaction (Fig. 3). Although some evidence of products within the 47-kDa range has been seen (Fig. 3, lanes 3 to 8), these bands were always faint. What factors may cause this difference? First, while both groups used the same immunoglobulin substrates, we chose to use commercially available proteins distributed in a lyophilized form. In contrast, Loomes et al. prepared immunoglobulin substrates fresh from serum and colostrum (46). It is possible that freshly prepared immunoglobulins retain a conformation required for the specificity of the metalloprotease, whereas lyophilization may cause a change in immunoglobulin conformation leading to a lack of specificity. It is also feasible that in purifying immunoglobulins, additional substances which aid in determining site specificity may have been copurified.

Another difference between the present work and that of Loomes et al. (46) is in the use of ^{125}I-labeled immunoglobulins as substrates. Labeling by chloramine T iodination may have altered the immunoglobulin molecule in such a way that sites normally attacked by the metalloprotease were blocked, thus limiting site availability and resulting in the production of two fragments. Interestingly, in earlier work reported by Milazzo and Delisle (48), an extracellular protease that hydrolyzes myeloma IgA to small fragments was isolated from *P. mirabilis*, an observation that is in agreement with our data. Such reports strengthen our finding. Moreover, it is possible that the discrepancy in proteolysis products is due to multiple extracellular proteases with similar substrate specificity; however, studies by Senior et al. (58, 59) reported the observation of only a single protease activity in strains of *P. mirabilis*. If this is true, as suggested earlier to explain the difference in protein size, our strain of *P. mirabilis* may be defective in one or more of these additional activities. We are exploring this possibility.

The *P. mirabilis* ZapA metalloprotease is very similar in many respects to the proteases of *P. aeruginosa* and *S. marcescens*, both of which degrade IgA and IgG (25, 51), and to the proteases of *E. chrysanthemi* (42). Together with ZapA, these metalloproteases constitute the serralysin family of proteases and share remarkable conservation of functional domains (>50% identity) and physiological characteristics. The serralysin family of proteases can be grouped with the snake venom proteases and matrix proteases in a larger family of the so-called metzincins characterized by the motif HEXXHXXG XXHZ, where the H residues are Zn ligands, Z is a proline (9), and a conserved methionine is located 30 to 60 residues from the C terminus (residue 412 of ZapA, located 79 residues from the end of the protein). The serralysin family is in turn a member of a larger group of virulence proteins from pathogenic *Actinobacillus* (*Haemophilus*) (18, 40, 41), *Bordetella* (16), enterohemorrhagic *E. coli* (33), *Neisseria* (64), *Pasteurella* (20), and *P. fluorescens* (27, 63) strains.

The proteases of the serralysin family are members of the ABC superfamily of prokaryotic and eukaryotic transporters (34, 54, 66). In these systems, three transport proteins probably combine to form zones of adhesion between the inner and outer membranes through which the proteins are secreted (23, 35). The proteins secreted by this system have common characteristics. First, they do not possess an N-terminal signal sequence, but they do contain a C-terminal targeting signal that is essential for secretion (22). The C-terminal targeting sequence ends with the conserved four-amino-acid motif DXXX (30). The Xs are hydrophobic amino acids and in the case of ZapA and PrtSM are DFIV (52). The COOH-terminal secretion mechanism is widely distributed among bacteria (65). Second, they do not contain any universally conserved primary structural elements; however, they all appear to contain a conserved putative amphipathic α-helix (29). Third, all of these proteins contain several (i.e., 4 to 13) repeats of the consensus sequence GGXGXD near the C-terminal secretion signal. Repetition of this motif is a characteristic feature of this secretion mechanism.

Our biochemical and deduced amino acid data support the conclusion that ZapA is a member of the serralysin family of the ATP-dependent ABC transporter protease superfamily. The strong homology between ZapA and other well-characterized proteases of this group is one means of judging this. Furthermore, preliminary evidence is provided for at least two additional ORFs upstream of *zapA* having sequences that are homologous to other members of the transporter portion of this family, e.g., *S. marcescens* PrtD and PrtE. A gene encoding the third protein of the ABC transporter, homologous to PrtF, has not been found, although its presence can be hypothesized by the minimum requirements of the ABC protease transporter system (27, 34). There is sufficient space in the area delineated by the Tn*5* insertions producing a Prt^- phenotype to encode for four proteins (ZapA plus three ABC transporters). Alternatively, the clone may not contain a gene encoding PrtE, whose function may be complemented by *E. coli* TolC (44). This issue will be clarified when the nucleotide sequencing and analysis of the upstream region are completed and the plasmid is introduced into a $TolC^-$ *E. coli* (unpublished data).

What is the relevance of the ZapA metalloprotease to UTI and to swarmer cell differentiation? Allison et al. (1, 4) have demonstrated that a number of virulence factors expressed by *P. mirabilis*, including a protease activity, are coordinately regulated during swarmer cell differentiation and required for invasion and colonization of host tissue. For this reason, it is a widely held hypothesis that differentiation to a swarmer cell is a prerequisite for *P. mirabilis* UTI (49). The role of microbial IgA proteases in pathogenesis has been difficult to assess because of their limited specificity for IgA of humans and a few higher primates (38). However, characterization of ZapA in the present report has demonstrated that this enzyme has specificity for mouse IgA (as well as human immunoglobulin) and thus provides a means to assess the role of this virulence factor in a mouse model. The well-characterized mouse model of ascending UTI has been used successfully to assess other virulence factors of *P. mirabilis* (36), and for this purpose, $ZapA^-$ mutants of *P. mirabilis* are presently being constructed. Moreover, such $ZapA^-$ mutants will also be useful in determining the role of this protease in swarmer cell differentiation, motility, and behavior. Preliminary evidence obtained by rescreening a bank of Tn*5* mutants in *P. mirabilis* with defects in swarming motility and behavior (12) has revealed that several are also defective in expression of ZapA (data not shown). Additional studies of these mutants will be required to understand the significance of the metalloprotease function and its relationship to swarmer cell differentiation and motile behavior.

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